(REV 10/01) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 36 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: MBP-009XX

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 10/049975

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/08116	18 August 2000 (18.08.00)	20 August 1999 (20.08.99)

TITLE OF INVENTION
METHOD FOR THE DETERMINATION OF SUBSTANCES USING THE EVANESCENCE FIELD METHOD

APPLICANT(S) FOR DO/EO/US
Manfred Schawaller, Gerald Quapil

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2)) **in German**
   a. ☐ is attached hereto (required only if not transmitted by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(3)).
   a. ☐ is attached hereto.
   b. ☐ had been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 20. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment. **(with attached German language Claims 1-26 from IPER)**
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☒ Other items or information:
    FORMAL DRAWINGS (6 sheets)
    Annex to the International Preliminary Examination Report in German (consisting of 4 sheets of claim 1-26).

Express Mail Number
EV 009952304 US

10/049975, 100102

JC13 Rec'd PCT/PTO 19 FEB 2002

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
10/049975	PCT/EP00/08116	MBP-009XX

21. ☒ The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1,040.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 890.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 0	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	6 - 20 =	0	X **$18.00**	$ 0	
Independent claims	1 - 3 =	0	X **$84.00**	$ 0	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			**+$280.00**	$ 0	
TOTAL OF ABOVE CALCULATIONS =				$ 890.00	
☒ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				$ 445.00	
SUBTOTAL =				$ 445.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 Months from the earliest claimed priority date (37 CFR 1.492(f)).				$ --	
TOTAL NATIONAL FEE =				**$ 445.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property				$	
TOTAL FEES ENCLOSED =				$	
				Amount to be Refunded:	$
				Charged:	$

a. ☒ A check in the amount of $ 445.00 to cover the above fees is enclosed. A check in the amount of $_____ is enclosed for the assignment recordation fee.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-0804 . A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

Customer Number 207
SEND ALL CORRESPONDENCE TO:

Weingarten, Schurgin, Gagnebin & Lebovici LLP
Ten Post Office Square
Boston, Massachusetts 02109

SIGNATURE

NAME: Charles L. Gagnebin III
REGISTRATION NUMBER: 25,467

Date: 2-19-2

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application	:	MANFRED SCHAWALLER, ET AL.
Application No.	:	10/049,975
Filed	:	February 19, 2002
For	:	METHOD FOR THE DETERMINATION OF SUBSTANCES USING THE EVANESCENCE FIELD METHOD
Examiner	:	
Attorney's Docket	:	MBP-009XX

Group Art Unit:

* *

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, Washington, D.C. 20231 on _____.

By:_____
Charles L. Gagnebin III
Registration No. 25,467
Attorney for Applicant(s)

* *

SUPPLEMENTAL PRELIMINARY AMENDMENT

BOX PCT
Commissioner for Patents
Washington, D.C. 20231

Sir:

Submitted for examination is the attached English translation of the claims that were amended by the International Preliminary Examination Report dated 26 November 2001.

Kindly enter the following Supplemental Preliminary Amendment in the above-identified application:

Express Mail Number
EV 044743055 US

WEINGARTEN, SCHURGIN,
GAGNEBIN & LEBOVICI LLP
TEL. (617) 542-2290
FAX. (617) 451-0313

Application No.: 10/049,975
Filed: February 19, 2002
Group Art Unit:

In the Amended Claims of the International Preliminary Examination Report (attached) dated 26 November 2001, please amend the Claims to read as follows:

Please add the following new claims 27-55:

27. The method according to claim 1, wherein the substance being assayed includes a biologically active substance, which is selected from the group of hormones, proteins, viruses, bacteria, pharmaceuticals and toxins.

28. The method according to claim 1, wherein the substance being assayed includes a protein, preferably an antigen or an antibody.

29. The method according to claim 1, wherein the compound containing fluorophor has a fluorescing compound and a binding site for the substance being assayed.

30. The method according to claim 1, wherein fluorescing proteins and/or low-molecular fluorescing chemical compounds are used as the fluorophor.

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31. The method according to claim 30, wherein phycobili proteins, such as allophycocyanine (APC), Cryptofluor Crimson or Cryptofluor Red are used as fluorescing proteins.

32. The method according to claim 31, wherein Cy5 or BODIFY are used as low-molecular fluorescing compounds.

33. The method according to claim 1, wherein at least one fluorophor that absorbs in a wavelength range from 600 to 700 nm is used.

34. The method according to claim 1, wherein at least one phosphorescing compound is used as the fluorophor.

35. The method according to claim 1, wherein a mixture of dyes that absorb in the absorption and/or emission range of the fluorophor is used.

36. The method according to claim 1, wherein at least one dye that absorbs in a wavelength range form 600 to 700 nm is used.

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37. The method according to claim 36, wherein Brilliant Blue FCF in a concentration of at least 0.001 mM is used as the at least one dye.

38. Cuvette or microtiter plate for use in the method according to claim 1 that have at least one reaction partner for the substance being assayed bonded to a surface, whereby the cuvette contains a plastic.

39. The cuvette or microtiter plate according to claim 38, whereby the at least one reaction partner R1 comes in lyophilized form.

40. The cuvette or microtiter plate according to claim 38, whereby the cuvette includes polystyrene, polypropylene, polyethylene, polyacrylnitrile, polymethylmethacrylate, polycycloolefin, polyethylene terephthalate and/or mixtures thereof.

41. The cuvette or microtiter plate according to claim 38, whereby the cuvette or microtiter plate is one-piece.

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42. The cuvette according to claim 38, whereby the cuvette has a reaction volume of 1 to 400 μl.

43. A solution containing at least one compound containing fluorophor, at least one dye and, if necessary, a reaction partner R2 for use in the method according to claim 1.

44. A kit for use in the method according to claim 1, including at least one plastic containing cuvette or microtiter plate having a reaction partner for a substance to be assayed.

45. The use of the method according to claim 1 to determine reaction kinetics of immunologic reactions.

46. The use of the method according to claim 1 in medical or veterinary medical diagnostics, food analysis, environmental analysis or analysis of fermentation processes.

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47. The method according to claim 27, wherein:

the substance being assayed includes a protein, preferably an antigen or an antibody;

the compound containing fluorophor has a fluorescing compound and a binding site for the substance being assayed;

fluorescing proteins and/or low-molecular fluorescing chemical compounds are used as the fluorophor;

phycobili proteins, such as allophycocyanine (APC), Cryptofluor Crimson or Cryptofluor Red are used as fluorescing proteins;

Cy5 or BODIFY are used as low-molecular fluorescing compounds;

fluorophor that absorbs in a wavelength range from 600 to 700 nm is used;

at least one phosphorescing compound is used as the fluorophor;

a mixture of dyes that absorb in the absorption and/or emission range of the fluorophor is used;

at least one dye that absorbs in a wavelength range form 600 to 700 nm is used;

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Brilliant Blue FCF in a concentration of at least 0.001 mM is used as the at least one dye.

48. The cuvette or microtiter plate according to claim 38, wherein:

the at least one reaction partner R1 comes in lyophilized form;

the cuvette includes polystyrene, polypropylene, polyethylene, polyacrylnitrile, polymethylmethacrylate, polycycloolefin, polyethylene terephthalate and/or mixtures thereof;

the cuvette or microtiter plate is one-piece;

the cuvette has a reaction volume of 1 to 400 μl.

49. A solution containing at least one compound containing fluorophor, at least one dye and, if necessary, a reaction partner R2 for use in the method according to claim 47.

50. A solution containing at least one compound containing fluorophor, at least one dye and, if necessary, a reaction partner R2 for use in the method according to claim 48.

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51. A kit according to claim 44 having at least one phosphor solution.

52. The use of the method according to claim 47 to determine reaction kinetics of immunologic reactions.

53. The use of the method according to claim 48 to determine reaction kinetics of immunologic reactions.

54. The use of the method according to claim 47 in medical or veterinary medical diagnostics, food analysis, environmental analysis or analysis of fermentation processes.

55. The use of the method according to claim 48 in medical or veterinary medical diagnostics, food analysis, environmental analysis or analysis of fermentation processes.

REMARKS

The English translation of PCT/EP00/08116 application and the English translation of the International Preliminary

WEINGARTEN, SCHURGIN,
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Application No.: 10/049,975
Filed: February 19, 2002
Group Art Unit:

Examination Report ("IPER") claims dated 26 November 2001 are being filed concurrently with this Supplemenetal Preliminary Amendment. Note that IPER claims 7-26 (in German) had previously been cancelled by the Preliminary Amendment filed with the application; IPER claims 1-6 remained unchanged.

Claims 1-6 remain unchanged; and new claims 27-55 have been added, adding back the subject matter of the previously cancelled claims 7-26. Kindly calculate the filing fee based on the amended claims presented herewith.

The Examiner is encouraged to telephone the undersigned attorney to discuss any matter which would expedite allowance of the present application.

Respectfully submitted,

MANFRED SCHAWALLER, ET AL.

By: _____
Charles L. Gagnebin III
Registration No. 25,467
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CLG/mc/267217-1

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application	:	MANFRED SCHAWALLER, ET AL.
Application No.	:	
Filed	:	Herewith
For	:	METHOD FOR THE DETERMINATION OF SUBSTANCES USING THE EVANESCENCE FIELD METHOD
Examiner	:	
Attorney's Docket	:	MBP-009XX

Group Art Unit:

* *

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents, Washington, D.C. 20231 on _____.

By:_____
Charles L. Gagnebin III
Registration No. 25,467
Attorney for Applicant(s)

* *

PRELIMINARY AMENDMENT

BOX PCT
Commissioner for Patents
Washington, D.C. 20231

Sir:

Kindly enter the following Preliminary Amendment in the above-identified application:

Please enter the amended claims (attached, in German language) of the International Preliminary Examination Report dated 26 November 2001.

Express Mail Number
EV 009952304 US

WEINGARTEN, SCHURGIN,
GAGNEBIN & LEBOVICI LLP
TEL. (617) 542-2290
FAX. (617) 451-0313

Attorney Docket No. MBP-009XX
Filed: Herewith
Group Art Unit:

In the Amended Claims of the International Preliminary Examination Report dated 26 November 2001:

Please cancel claims 7-26.

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Attorney Docket No. MBP-009XX
Filed: Herewith
Group Art Unit:

REMARKS

The English translation of International application No. PCT/EP00/08116 will be filed in due course, as well as the English translation of the amended claims of the International Preliminary Examination Report ("IPER"). Note that IPER claims 7-26 (multiple dependent and related claims) have been cancelled; and claims 1-6 remain unchanged. Kindly calculate the filing fee based on the amended claims.

The Examiner is encouraged to telephone the undersigned attorney to discuss any matter which would expedite allowance of the present application.

Respectfully submitted,

MANFRED SCHAWALLER, ET AL.

By: _____
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Date: 2-19-3

CLG/mc/267092-1

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In re application	:	MANFRED SCHAWALLER, ET AL.
Application No.	:	
Filed	:	Herewith
For	:	METHOD FOR THE DETERMINATION OF SUBSTANCES USING THE EVANESCENCE FIELD METHOD
Examiner	:	
Attorney's Docket	:	MBP-009XX

ANNEX TO

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

of PCT/EP00/08116

DATED 26 November 2001

IN GERMAN LANGUAGE

CONSISTING OF: 4 PAGES OF CLAIMS 1-26

Amtl. Aktenzeichen: PCT/EP00/08116
Anmelder: Stiftung für Diagnostische Forschung
"Verfahren zur Bestimmung von Substanzen mittels der Evaneszenzfe ldmethode"
Unser Zeichen: D 2724 - py / ml

Ansprüche

1. Verfahren zur Bestimmung von Substanzen, umfassend die Schritte
   - Bereitstellen einer Oberfläche, welche mindestens einen Reaktionspartner R^1 an der Oberfläche gebunden umfaßt,
   - Kontaktieren der Oberfläche mit einer Lösung, welche mindestens die zu bestimmende Substanz, mindestens eine Fluorophor-haltige Verbindung und mindestens einen Farbstoff, welcher im Absorptions- und Emissionsbereich des Fluorophors absorbiert, umfaßt,
     worin sich an dem Reaktionspartner R^1 auf der Oberfläche ein Komplex ausbildet und worin dieser Komplex neben dem Reaktionspartner R^1 mindestens die zu bestimmenden Substanz und die mindestens eine Fluorophor-haltige Verbindung umfaßt, und
   - Anregen des auf der Oberfläche gebundenen Fluorophors durch das Evaneszenzfeld einer Lichtquelle und Messen der erzeugten Fluoreszenz.

2. Verfahren nach Anspruch 1, worin die zu bestimmende Substanz als Reaktionspartner R^2 an den Reaktionspartner R^1 auf der Oberfläche bindet.

3. Verfahren nach Anspruch 2, worin der an der Oberfläche gebundene Reaktionspartner R^1 ein Antigen oder ein Antikörper ist.

4. Verfahren nach Anspruch 1, worin ein Reaktionspartner R^2 die zu bestimmende Substanz umfaßt und an den Reaktionspartner R^1 auf der Oberfläche bindet.

5. Verfahren nach Anspruch 1, worin eine weitere Verbindung, welche eine Bindungsstelle für die zu bestimmende Substanz aufweist und einen Reaktions-

partner R^2 enthält, an den Reaktionspartner R^1 auf der Oberfläche bindet.

6. Verfahren nach Anspruch 5, worin der Reaktionspartner R^1 Avidin oder Streptavidin umfaßt und der Reaktionspartner R^2 Biotin und eine Bindungsstelle für die zu bestimmende Substanz umfaßt.

7. Verfahren nach einem der vorangehenden Ansprüche, worin die zu bestimmende Substanz eine biologisch aktive Substanz umfaßt, welche aus der Gruppe Hormone, Proteine, Viren, Bakterien, Pharmazeutika und Toxine ausgewählt ist.

8. Verfahren nach einem der vorangehenden Ansprüche, worin die zu bestimmende Substanz ein Protein, vorzugsweise ein Antigen oder einen Antikörper, umfaßt.

9. Verfahren nach einem der vorangehenden Ansprüche, worin die Fluorophorhaltige Verbindung eine fluoreszierende Verbindung und eine Bindungsstelle für die zu bestimmende Substanz aufweist.

10. Verfahren nach einem der vorangehenden Ansprüche, worin als Fluorophor fluoreszierende Proteine und/oder niedermolekulare fluoreszierende chemische Verbindungen verwendet werden.

11. Verfahren nach Anspruch 10, worin als fluoreszierende Proteine Phycobiliproteine, wie Allophycocyanin (APC), Cryptofluor Crimson oder Cryptofluor Red, verwendet werden.

12. Verfahren nach Anspuch 11, worin als niedermolekulare fluoreszierende Verbindungen Cy5 oder BODIPY verwendet werden.

13. Verfahren nach einem der vorangehenden Ansprüche, worin mindestens ein Fluorophor verwendet wird, welcher in einem Wellenlängenbereich von 600 bis 700 nm absorbiert.

14. Verfahren nach einem der vorangehenden Ansprüche, worin mindestens eine phosphoreszierende Verbindung als Fluorophor verwendet wird.

15. Verfahren nach einem der vorangehenden Ansprüche, worin eine Mischung von Farbstoffen verwendet wird, welche im Absorptions- und/oder Emmisionsbereich des Fluorophors absorbieren.

16. Verfahren nach einem der vorangehenden Ansprpüche, worin mindestens ein Farbstoff verwendet wird, welcher in einem Wellenlängenbereich von 600 bis 700 nm absorbiert.

17. Verfahren nach Anspruch 16, worin als der mindestens eine Farbstoff Brilliantblau FCF in einer Konzentration von mindestens 0,001 mM verwendet wird.

18. Küvette oder Mikrotiterplatte zur Verwendung in dem Verfahren nach einem der Ansprüche 1 bis 17, welche mindestens einen Reaktionspartner für die zu bestimmende Substanz an einer Oberfläche gebunden umfaßt, wobei die Küvette einen Kunststoff umfaßt.

19. Küvette oder Mikrotiterplatte nach Anspruch 18, wobei der mindestens eine Reaktionspartner R^1 in lyophilisierter Form vorliegt.

20. Küvette oder Mikrotiterplatte nach Anspruch 18 oder 19, wobei die Küvette Polystyrol, Polypropylen, Polyethylen, Polyacrylnitril, Polymethylmehtacrylat, Polycycloolefin, Polyethylenterephthalat und/oder Mischungen derselben umfaßt.

21. Küvette oder Mikrotiterplatte nach einem der Ansprüche 18 bis 20, wobei die Küvette oder Mikrotiterplatte einteilig ist.

22. Küvette nach einem der Ansprüche 18 bis 21, wobei die Küvette ein Reakti-

onsvolumen von 1 bis 400 µl aufweist.

23. Lösung, enthaltend mindestens eine Fluorophor-haltige Verbindung, mindestens einen Farbstoff und gegebenenfalls einen Reaktionspartner R^2 zur Verwendung in einem Verfahren nach einem der Ansprüche 1 bis 17.

24. Kit zur Verwendung in einem Verfahren nach einem der Ansprüche 1 bis 17, umfassend mindestens eine Küvette oder Mikrotiterplatte nach einem der Ansprüche 18 bis 22, und/oder mindestens eine Lösung nach Anspruch 23.

25. Verwendung des Verfahrens nach einem der Ansprüche 1 bis 17, zur Bestimmung von Reaktionskinetiken immunologischer Reaktionen.

26. Verwendung des Verfahrens nach einem der Ansprüche 1 bis 17 in der medizinischen oder veterinärmedizinischen Diagnostik, der Lebensmittelanalytik, der Umweltanalytik oder der Analytik von Fermentationsprozessen.

"Method of Assaying Substances Using the Evanescence Field Method"

Description

The present invention concerns a method for assaying substances based on the evanescence field method and a cuvette, a microtiter plate, a solution and a kit for use with the method in the invention. This invention can be used particularly in diagnosis and analysis.

Medical diagnostics, especially immunological diagnostics, is largely based on the ELISA (Enzyme-Linked-Immunoabsorbent Assay). A recent review of immune assays can be found in Hage, Anal. Chem. 71 (1999), 294R-304R. An ELISA test is used to determine the concentration of antigens or antibodies. The substance being studied (for example, an antigen) is first placed in contact with a solid substrate to which a specific reaction partner for the substance being studied is first coupled (for example, an antibody). By binding the substance being studied to the reaction partner coupled to the substrate, the substance being studied is concentrated on the solid substrate. Then, a second reaction partner (for example, another antibody) for the substance being studied is placed in contact with the substrate, and this reaction partner is marked with an enzyme, which allows colorimetric detection. When this second reaction partner reacts with the substance being studied coupled to the surface of the substrate, a colored product is produced that can be evaluated optically. Standardized plastic plates, frequently made of polystyrene, with 96 wells are mostly used as the solid phase. The surface of the plastic wells binds proteins in the nanogram range through absorption in a quantity sufficient for immunological detection. There are several ways of marking the second reaction partner, which is mostly an immunoglobulin, with an enzyme. Markers currently used are peroxidase or alkaline phosphatase.

ELISAs give very good results in terms of sensitivity and specificity, and the detection limits that can be reached are in the nanogram range or below it. There is a wide variety of embodiments of assays that are based on this principle. With it, antigens or antibodies can be detected, depending on what the question is.

However, a major disadvantage of the ELISA is handling the test, since different reagents are added to the wells one after another and must be removed again. Ten or more pipetting, washing and incubation steps in all may be necessary. So ELISAs are very time-consuming and labor-intensive, and must be done by specially trained personnel with great care. Another disadvantage of the ELISA is the time it takes for all the incubation and washing steps for an assay or test, which normally lasts one hour or more.

With the evanescence field method, the interaction of biomolecules, for example, on a surface can be observed directly. Here, the interaction of reactants in solution is measured with a solid matrix surface. It is possible to measure the binding of the ligands physically as "surface plasmon resonance" in "real time."

The advantages compared to an ELISA are the elimination of other pipetting steps after the addition of the reagents and the elimination of the waiting steps. In the past, expensive apparatuses and multi-layer sensor chips with special surface chemistry were needed for such measurements. These disadvantages prevent the method from being used in routine diagnostics.

Thus, the technical problem underlying the present invention is based on providing a method of assaying substances, especially biologically active substances in which the washing and pipetting steps usual with an ELISA can be avoided as much as possible, and the incubation times can be reduced. This method should also require inexpensive sensor chips and cuvettes that are easy to produce and available.

The embodiments in the claims provide a solution to the above technical problem.

In particular, the invention provides a method of assaying substances that has the following steps:

- providing a surface that has at least one reaction partner R1 for a reaction partner R2 bonded to the surface,
- placing in contact with the surface a solution that contains at least the substance being assayed, at least one compound containing fluorophor and at least one dye that absorbs in the absorption and/or emissions range of the fluorophor, wherein a complex forms at reaction partner R1 on the surface by means of reaction partner R2 and wherein that complex includes, besides reaction partner R1 at least the substance being studied and the compound containing at least one fluorophor, and
- exciting the fluorophor bonded to the surface by the evanescence field of a light source and measuring the fluorescence produced.

The figures show:

Figure 1 is a schematic view of an embodiment of the cuvette in the invention and the method in the invention in one form of embodiment.

Figure 2 shows a schematic view of another embodiment of the method in the invention.

Figure 3 shows changes in the intensity of electromagnetic radiation in a dye solution according to Lambert-Beer's Law.

Figure 4 shows a double logarithmic view of changes in the intensity as a function of the penetration depth of an evanescent wave and a wave weakened by absorption according to Lambert-Beer's Law.

Figure 5 shows the absorption spectra of a series of dyes.

Figure 6 shows the determination of the optimum concentration of a dye for use in the method in the invention.

Figure 7 shows a reaction kinetic measured by the method in the invention for the absorption of a protein on reaction partner R1 bonded to the surface.

Figure 8 shows a comparative measurement for the reaction kinetics in Figure 7 measured in Figure 7. However, the surface in this comparative example was not coated with reaction partner R1 for the protein.

According to the invention, first a surface is prepared that has at least one reaction partner R1 bonded or immobilized. Bonded preferably means that the reaction partner R1 is adhered to the surface by absorption (direct absorption). But the reaction partner R1 can also be bonded to the surface via a bridge element, for example a protein, such as an antibody or an antigen. The reaction partner R1 can also be bonded to the surface by a covalent bond. This can be produced with an acrylate surface by conversion with a carbodi-imide, for example. The term "bonded" in the sense of the invention means adhesion of a reaction partner or connection to a surface or to another reaction partner and/or connection, and includes both covalent and non-covalent interactions, like for example interactions based on ionic, polar or non-polar interactions.

Reaction partner R1 can be placed on the surface by a common method. For example, a protein serving as reaction partner R1 can be coated on the surface. Reaction partner R1 can preferably be bonded to the surface by absorption or by a covalent bond. After this step, the surface is preferably treated with another solution, and places on the surface not adhered to reaction partner R1 are blocked or will be blocked, for example by another protein that basically does not react with the components contained in the solution to make contact. The above surface is the inside of a concave container, like a cuvette or a well of a microtiter plate, for example.

According to the invention, reaction partner R1 bonded to the surface can form a complex by means of reaction partner R2 on the surface, whereby this complex includes, besides reaction partner R1, at least the substance being assayed and the compound containing at least one fluorophor. With reaction partner R1 bonded to the surface, the complex with the substance being assayed is "anchored" to the surface, i.e., fixed and can at the same time be detected by marking it with a compound containing the fluorophor.

According to the invention, a "complex" or "conjugate" is understood to be a molecular coupling or bonding between two or more preferably chemical or biochemical substances. The complex is preferably formed by means of selective and/or specific conversions, especially preferred by antigen-antibody reactions. According to the invention, the term "conversion" includes both covalent and non-covalent interactions of two or more reaction partners, wherein both types of interaction can take place one after another within a complex or conjugate. Non-covalent interaction can mean, for example, Van der Waals interaction, polar and/or ionic interaction of reaction partners. The term "reaction partner" means a compound with an affinity for another substance in this invention.

According to the invention, the complex includes, besides reaction partner R1, at least the substance being assayed and the compound containing at least one fluorophor.

The following are ways, inter alia, of binding this complex to reaction partner R1 by means of reaction partner R2:

(1) The substance being assayed is itself reaction partner R2.

(2) The substance being assayed contains reaction partner R2, i.e., reaction partner R2 is a part of the structure of the substance being assayed.

(3) The substance being assayed has an affinity or binding site for reaction partner R2. After reaction partner R1 is bonded to the substance being assayed, case (2) can therefore apply.

(4) Another compound contains reaction partner R2 or has an affinity for reaction partner R2, whereby this other compound also contains at least one binding site for the substance being assayed. In this case, the other compound, the substance being assayed and the reaction partner R2 can be in the solution as a conjugate or complex (all or only individually) or the conjugate is formed in the solution.

Preferred embodiments of these cases (1) to (4) will be described in greater detail below.

In one preferred embodiment of the method in the invention, the substance being assayed can itself have an affinity for reaction partner R1 on the surface and can therefore bind directly with that reaction partner R1. In this embodiment, the substance being assayed, as reaction partner R2, can bind to reaction partner R1 on the surface. When the substance being assayed is an antibody, for example, an antigen specific for that antibody can be placed on the surface, or vice versa.

Figure 1 shows a schematic view of an embodiment of the cuvette in the invention, and the method in the invention according to the embodiment above. The cuvette 1 has a well 2, whose surface 3 contains reaction partner R1 4 bonded for the protein being assayed. The well 2 also holds the solution 5 to be placed in contact with the surface 3, which, in this embodiment, is a dye 6 and the substance 7 being assayed, which already exists as a conjugate with the compound containing fluorophor. The substance being assayed reacts with reaction partner R1 bonded to the surface into a complex 9 on the surface 3. For example, with a laser diode 12, a beam of light 10 is projected onto the bottom of the surface 3,

which is totally reflected on the surface of the phase boundary 11. That way, an evanescence field 13 is formed over the surface 3 in which there is basically only fluorophor bonded to the surface in complex 9. In contrast to the schematic view in Figure 1, the evanescence field usually does not extend over the whole width of the base of the cuvette. For example, the evanescence field can have an expansion of roughly 1 mm^2. By exciting the fluorophor through the evanescence field 13, the fluorophors bonded to the surface emit photons 14, which can be enhanced by means of a photomultiplier 15, for example, and can be measured. The fluorescence of the volume 16 is basically suppressed by the presence of the dye 6.

In another preferred embodiment, the substance being assayed itself has (basically) no affinity or only a small affinity for reaction partner R1 on the surface. In this case, the solution to be placed in contact with the surface contains, for example, another compound that contains reaction partner R2 and a binding site for the substance being assayed. Reaction partner R2 can bind to reaction partner R1 on the surface and thus fixes the substance being assayed indirectly to the surface. This other connection serves as a bridge element between the substance being assayed and reaction partner R1 on the surface. For example, avidin can be present as reaction partner R1 on the surface. The other compound then contains, besides a binding site for the substance being assayed, for example biotin, which can bind to the avidin bonded to the surface. This embodiment has the advantage that a surface coated with avidin, unlike many antibodies and antigens, can be lyophilized and dried or is very stable lyophilized. In addition, the avidin/biotin system has a very high dissociation constant K_D. It is also possible for a series of different assays to be done on a surface coated with avidin and to assay only the other compound, which is placed in contact with the surface with the solution, on the substance being assayed.

Figure 2 shows this embodiment of the method in the invention schematically. In the solution placed in contact with the surface are, next to one another, the substance being assayed 20, a dye 22, a compound 24 containing fluorophor and another compound 26. Reaction partner R1 28 is bonded to the surface. The other compound and the compound containing fluorophor are absorbed on the substance being assayed (conjugate 30), and the conjugate 30 is bonded via reaction partner R2 present in the other compound 26 on reaction partner R1 28 on the surface to complex 32. Thus, complex 32, which includes compound 24 containing fluorophor, is bonded to the surface and can be assayed by measuring the fluorescence in the evanescence field 34.

For this embodiment of the method of the invention, besides the avidin (or streptavidin)/biotin system, all ligands or ligand-binding systems in which proteins, for example, have selective and/or specific binding sites for one or more ligands, like for example histidine, histidine tags, lectin and/or digoxigernin, and naturally antigen/antibody systems are suitable.

The solution in the invention that is placed in contact with the surface also contains at least one compound containing fluorophor. According to the invention, a fluorophor is understood as a

fluorescing compound, such as a fluorescent dye. Fluorescing proteins and/or low-molecular fluorescing chemical compounds are preferred. According to the invention, phycobili proteins, such as Allophycocyanine (APC), Cryptofluor Crimson or Cryptofluor Red can be used as fluorescing proteins. Cy5 or BODIPY (4,4-diluor-4-bora-3a,4a-diaza-s-indazene-fluorophore) [sic] can be cited as examples of low-molecular fluorescing compounds. Fluorescing dyes with an absorption range from 600 to 700 nm are preferred.

Instead of a fluorophor, a fluorophor precursor compound can be used, from which the fluorophor is released before the measurement process, for example, by changing the pH value or by splitting a protective group.

According to the invention, the term fluorophor also includes phosphorescing compounds. If such a phosphorescing compound is used as a fluorophor, the phosphorescence radiated, which is staggered in time from the excitation, is determined. Thus, it is possible to separate the radiation time from the measurement time.

This compound containing fluorophor also has a binding site for the substance being assayed. For example, the fluorophor can come bonded to an antibody. This antibody containing fluorophor can preferably react as an antigen in an antigen-antibody reaction with the substance being assayed, for example a protein.

In another embodiment, the substance being assayed itself comes as a compound containing fluorophor. In this embodiment, competitive assays are done, which are characterized especially by a low detection limit.

With the method in the invention, a wide variety of substances can be detected. The method is especially suitable for assaying biologically active substances, like hormones, proteins like antigens, antibodies or haptenes, pharmaceuticals, viruses, bacteria, etc. But the method can also be used to detect environmental poisons, toxins, etc.

It is especially preferred for the substances being assayed to be detected by immunologic reactions.

According to the invention, a complex is formed of at least the first reaction partner R1, the substance being assayed and the compound containing fluorophor on the surface. Then, it is possible to measure the fluorophor bonded to the surface by exciting the evanescence field of a light source and measuring the fluorescence of the fluorophor.

When exciting the fluorophor bonded to the surface with an evanescence field, a beam of light is pointed at the bottom of the surface at an angle such that total reflection occurs at the cuvette/solution phase boundary. This forms an evanescence field above the surface in the solution, which can penetrate

up to several hundred nanometers into the fluid. According to this invention, an angle of incidence of at least 60° to 90° is preferred, so that an evanescence field at a height up to 400 nm, preferably 200 nm, and especially preferred 50 to 150 nm, is formed over the surface. Within this evanescence field, the beamed light may excite suitable fluorophors. The fluorescent light emitted is enhanced with a photomultiplier, for example, and evaluated.

Since only the fluorophor bonded to the surface is in the evanescence field, only this bonded fluorophor is optimally excited and emits photons. A compound that contains fluorophor and is not bound in the solution is not in the area of the evanescence field, is therefore basically not excited and also basically emits no photons. This arrangement thus allows quantitative determination of fluorophor bonded to the surface in the presence of fluorophor in the supernatant solution without a prior separation and/or washing step.

Monochromatic light can be used as the light source. Light should be used that has a wavelength that preferably does not interfere with the emission of the fluorophor and preferably intersects with the absorption band of the dye. A laser is especially preferred as a light source, whose light emits a wavelength of at least 635 nm. In particular, if the supernatant solution is a serum, lasers that emit wavelengths from 600 to 700 nm are preferred, since the serum's inherent fluorescence is roughly 580 nm.

In one embodiment of the invention, the addition of the fluorophor bonded to the surface can be measured directly (in real time) with a time-progressive reaction. Since the quantity of fluorophor bonded to the surface is directly proportional to the original amount of compound containing fluorophor, the method in the invention makes it possible to make a quantitative determination of reactants found in the solution in real time without other additional washing and/or pipetting steps.

Since the absorption coefficients and the emission properties of fluorophors are very good, the detection limits are small. After only a few minutes, reactions can be assessed qualitatively and/or quantitatively.

However, the scatter of the light beam in the cuvette, which is not ideal, poses a problem, even if physical measures are taken to reduce the scatter light. Due to scatter, light also gets into the volume in the cuvette and causes background fluorescence there. The term "volume" is understood in the present invention to be the liquid outside the evanescence field, which contains unbonded compounds containing fluorophor. The polarization of the light beam can also be turned in both plastic and glass cuvettes. This leads, in particular, to reflections of the excitation light during uncoupling, creating so-called vagabond light, which, along with volume and surface scatter effects, can result in excitation of the volume.

According to the invention, excitation of the fluorophor in the volume can be suppressed if the solution to be placed in contact with the surface has at least one dye added to it that has an absorption in the absorption and/or emissions range of the fluorophor.

A comparison of the penetration depth of evanescent waves and vagabond light shows that suppressing volume excitation by adding a dye works. Physically, the light absorption is described by Lambert-Beer's Law, whereby the intensity of the light decreases logarithmically with the distance due to absorption:

$$I[x] = I_0 Exp(-\alpha c x)$$

Whereby I_0 is the intensity of the light shining into the absorbing medium, I is the intensity of the light coming out of the absorbent medium, x is the thickness of the absorbing medium (layer thickness), α is the absorption coefficient and c the concentration of a dye in the solution. Figure 3 gives the changes in the intensity of a solution of an absorber dye with increasing layer thickness, showing the change in intensity for α = 100,000 Mol/(l x cm) and c = 20 mMol up to a depth of 1 mm. It must be recognized that over this distance, the scatter light is weakened to 1/100 of its initial intensity. Since the scatter light is predominantly coupled laterally, this weakening is enough to keep the volume signal and hence also the measurement uncertainty for the time-dependent signal of the reaction kinetics, on which this signal is overlapped, within practical usable limits.

Figure 4 shows a comparison of the penetration depths for the evanescent wave with the penetration depth of the light during absorption by a dye. This double logarithmic view shows the changes in intensity depending on the penetration depth of an evanescent wave (left) and a wave weakened by absorption (right). The ordinate is in the range from –2 to 0, i.e., from 1/100 to 1 log10 intensity. The parameters on which it is based are technically feasible values. Although damping the vagabond light permits much greater penetration depths than the light of the evanescent wave, volume excitation and/or emission can still be suppressed efficiently and basically quantitatively, as will be shown in the examples.

The decisive factor for the efficacy of the suppression is the geometric distance between that part of the surface of the cuvette from which light can reach the detector, and the penetration sites of the vagabond light in the volume.

As is clear from Figure 4, a distance in the range of one millimeter is enough to weaken scatter light of two magnitudes. This distance can be maintained simply by corresponding dimensioning of the cuvette.

The absorption of the dye added to the volume is coordinated with the absorption and/or emission range of the fluorophor in the invention. One individual dye or a mixture of dyes can be used. The absorption range of the fluorophor generally correlates with the wavelength of the light source used. It is not necessary that the dye have an absorption maximum in this spectral range; a shoulder in the

absorption spectrum can suffice. For example, if fluorophors like APC or Cy5 are used, the dye used can have an absorption between 600 nm and 700 nm, like for example Brilliant Blue FCF. The concentration of dye added depends on both the absorption coefficient of each dye in solution and the frequency of the light radiated. The concentration of dye can be adjusted, depending on the dye, so that the penetrating light can basically be absorbed within 1 mm above the surface. To determine the optimal concentration of dye, first the volume fluorescence and the fluorescence in the evanescence field, i.e., the surface fluorescence, are measured for various concentrations of dye (see Figure 6a). Then, the ratio of surface fluorescence to volume fluorescence is plotted against the concentration of dye (see Figure 6b). The maximum of curve 6b represents the optimum concentration of dye. According to the invention, "signal/noise ratio" is the ratio of surface fluorescence (signal) to volume fluorescence ("noise"). "Basically absorbed" can mean an intensity cancellation of 70%, preferably 80% and especially preferred at least 90%.

For example, when Brilliant Blue FCF is used as the dye, a concentration of 0.04 mM is enough to suppress far more than 95% of the volume fluorescence (see Table 4, Example 4). Since the necessary concentration of dye depends, inter alia, on the cuvette used, the measurement layout, etc., even smaller dye concentrations may suffice for an adequate signal/noise ratio. For example, the concentration of Brilliant Blue FCF is preferably at least 0.001 mM.

Comparative experiments, as shown in Figure 6a and 6b, have shown that the signal/noise ratio of 1.3:1 to up to 18.5:1 could be improved with the method in the invention.

The present invention also relates to a cuvette and a microtiter plate for the method in the invention. The cuvette preferably contains glass or a plastic, especially preferably a plastic, such as polystyrene, polypropylene, polyethylene, polyethylene terephthalate, polycycloolefin, polyacrylnitrile, polymethylmethacrylate and/or mixtures or blends of these plastics. In principle, any plastic that basically absorbs no light in the visible range is suitable. In one form of embodiment, the plastic can also be dyed light blue, for example, in order to filter out an emission caused by scatter light. Plastic cuvettes can be obtained inexpensively by injection molding and preferably have a reaction volume of 1 to 400 µl, and especially preferred 5 to 200 µl. Preferably, the cuvettes or microtiter plates in the invention are made in one piece. It can also be an advantage if the inside and/or emission surface, i.e., the surface from which the emitted beam comes out of the cuvette, is/are polished to a surface roughness of preferably 10 nm maximum.

The small dimension and low price make using the method in the invention feasible in routine diagnostics and analysis. In practical application, this type of cuvette or microtiter plate can be pre-prepared and sold commercially closed with a special label. The pre-preparation includes coating the surface of the cuvette or microtiter plate with the first reaction partner and, if necessary, then blocking the uncoated places. It is especially preferred if the coated cuvette or microtiter plate comes lyophilized or dried. The compound containing at least one dye and/or at least one fluorophor and/or the other

compound can come lyophilized and/or dried in a closed cuvette or microtiter plate, so that the substance being studied need only be added to the solution. Providing the cuvette or microtiter plate with a serial number makes it possible to have clear attribution of the manufacturing lot, the detection reaction and the sample at any time.

This invention also includes a solution that contains at least one compound containing a fluorophor, and/or at least one dye that absorbs in the absorption and/or emissions range of the fluorophor. The solution in the invention can also contain another compound that has at least one binding site for the substance being assayed and which includes reaction partner R2.

The invention also includes a kit, which can contain a cuvette or microtiter plate pre-prepared as described above, and/or solutions of at least one dye and at least one compound containing a fluorophor and, if necessary, another compound that has at least one binding site for the substance being assayed and reaction partner R2. The compound containing at least one dye and at least one fluorophor can come together in one solution and in two separate solutions.

This invention also relates to the use of the method in the invention for determining reaction kinetics, preferably immunologic reactions, as well as the use of the method in medical or veterinary medical diagnostics, food analysis, environmental analysis or analysis of fermentation processes.

Examples of specific applications that can be cited are detection of plant protection media, such as atrazine, in drinking water, detection of hormones in veal, detection of hormones like HCG and direct or indirect detection of viruses, such as Hepatitis S and HIV.

The invention will be further explained with reference to the examples below.

Example 1

In this example, the influence of the concentration of the fluorophor bonded to a protein and added is determined. In this measurement, the only fluorophor is the one bonded to the surface; unbonded fluorophor was washed away. A dye was not added to the solution.

a) Coating the Surface of a Cuvette with CACMAK

The surface of a cuvette was coated by leaving on the surface over night (ON) at room temperature (RT) 200 µl mouse IgGl, monoclonal antibody Ac1-20.4-2. (CACMAK; Progen Biotechnik GmbH, Heidelberg, Germany) 5 µg/ml in PBS+ (PBS+ = 100 mM PO_4, pH 7.5; 100 mM NaCl). Then the surface was washed four times with PBS (phosphate buffered saline) and treated with 1% BSA (bovine serum albumin) Miles Enhanced, PBS+ 300 µl, for one hour at RT.

b) Placing the Protein Being Assayed in Contact with the Surface

GAMAPC (a conjugate of allophycocyanin (APC) and crosslinked goat anti-mouse IgG (H+L); Molecular Probes, Leiden, Netherlands) in PBS+T (PBS+T = 100 mM PO_4, pH 7.5; 100 mM NaCl, 0.025v/v Tween 20) was left on the surface over night at RT. Then, it was washed five times with PBS, and 200 µl PBS+T was added, and the fluorescence was measured by the evanescence field method. The results are shown in Table 1.

Table 1

Concentration of coating solution of antigen CACMAK [µg/µl]	Concentration of GAMAPC [µg/µl]	Emission [photon counts/s]
0	10	3,000
5	10	120,000
5	3	60,000
5	1	18,000
5	0	3,000

The emission of photons dependent on the concentration of the fluorophor APC bonded to the surface was then found.

Example 2

End-point reaction with chip washing and fluorescence measurement. Only bonded fluorophor is present for the measurement.

a) Coating the Surface of a Cuvette

The surface of cuvettes was coated by leaving 200 µl of human serum 1:1000 in PBS+ at room temperature (RT) over night on the surface. Then the surface was washed four times with PBS and treated with 1% BSA (bovine serum albumin) Miles enhanced, PBS+, 300 µl for one hour at RT.

b) Placing the Protein Being Assayed in Contact with the Surface

Anti-human-IgG-Cy5 conjugate (Amersham Pharmacia Biotech, Dübendorf, Swtizerland) in PBS+T was left on the surface over night at RT. Then, it was washed five times with PBS, and 200 µl PBS+T was added and the fluorescence was measured. The results are shown in Table 2.

Table 2

Coated human serum antigen	Anti-human IgG-Cy5 conjugate	Emission [photon counts/s]
0	1:100	3,000
1:1000	1:100	7,000
1:1000	1:300	6,000
1:1000	1:1000	5,000
1:1000	0	3,000

In turn, it was possible to measure the emission of photons dependent on the concentration of the bonded fluorophor Cy5.

Example 3

In this example, the effectiveness of different dyes in reducing volume absorption is studied. The surface of the cuvette was not coated in this example, and only the reduction in fluorescence of the conjugate of protein and fluorophor in the solution was determined. The fluorophors in the volume of the reaction solution were excited by small amounts of scatter light and fluoresced. The absorption spectra of the dyes used are shown in Fig. 5.

GAMAPC 10 µg/ml in PBS+T is mixed with different dyes and the fluorescence is measured by volume excitation. The results are shown in Table 3.

Table 3

Dye Added	Absorption of Dye at 650 nm	Emission of Fluorophor [fluorescence counts/s]
Cuvette without fluorophor	-	3,300
No absorber dye	0.00	210,000
Brilliant Blue FCF[1] 0.25 mM	0.55	4,200
Amaranth[2] 1 mM	0.05	120,000
5% Supercook Blue[3]	0.30	4,400
5% Supercook Green[3]	0.10	23,000
5% Supercook Egg Yellow[3]	<0.04	190,000
5% Supercook Pink[3]	<0.04	200,000
5% Supercook Cochineal[3]	0.05	99,000

Notes:

1) Brilliant Blue FCF (Erioglaucine A), Fluka, Buchs, Switzerland
2) Amaranth, Fluka, Buchs, Switzerland
3) Supercook Food Colourings, Supercook, Leeds, Great Britain

It was thus found that when APC was used as fluorophor, dyes or mixtures of them that absorb between 600 and 700 nm reduce the volume fluorescence by absorption of the light that shines in and/or is emitted.

Example 4

In this example, the dependence of reducing volume fluorescence on the concentration of the dye Brilliant Blue FCF using APC as a fluorophor is studied.

a) Preparation of Cuvette

The cuvette is blocked with 1% BSA Miles enhanced, in PBS+ 300 µl for one hour at RT.

b) Placing the Solution Containing the Fluorophor and the Dye in Contact

GAMAPC (10 µg/ml) in PBS+T is mixed with Brilliant Blue FCF in different concentrations, and the fluorescence of the volume excitation is measured.

Table 4

Dye in PBS+T added	Concentration of dye [mM]	Emission of fluorophor [fluorescence counts/s]
None (only cuvette)	--	3,300
None (cuvette + GAMAPC)	--	106,000
GAMAPC + Brilliant Blue FCF[4]	0.02	26,000
GAMAPC + Brilliant Blue FCF[4]	0.04	9,000
GAMAPC + Brilliant Blue FCF[4]	0.08	4,000
GAMAPC + Brilliant Blue FCF[4]	0.16	4,000
GAMAPC + Brilliant Blue FCF[4]	0.32	4,000
GAMAPC + Brilliant Blue FCF[4]	0.63	4,000
GAMAPC + Brilliant Blue FCF[4]	1.25	4,000
GAMAPC + Brilliant Blue FCF[4]	2.50	4,000
GAMAPC + Brilliant Blue FCF[4]	5.0	4,000
GAMAPC + Brilliant Blue FCF[4]	10.0	4,000

Note: 4) Brilliant Blue FCF (Erioglaucine A), Fluka, Buchs, Switzerland.

The reduction in volume excitation depends on the concentration of Brilliant Blue dye in the volume. With Brilliant Blue FCF, at a concentration of only 0.04 mm and above, far more than 95% of the volume fluorescence is suppressed.

Example 5

In this example, the influence of the dye on the fluorescence of the fluorophor bonded to the surface is studied. End-point reaction with surface washing and fluorescence measurement. Only bonded fluorophor is measured.

A cuvette prepared as in Example 1a) is placed in contact with GAMAPC in PBS+T over night at RT and then washed five times with PBS.

After 200 µl of PBS+T is added, mixed with Brilliant blue FCF in different concentrations, the fluorescence of GAMAPC 10 µg/ml in PBS+T bonded to the surface of the cuvette and the fluorescence was measured by volume excitation.

Table 5

Dye in PBS+T added	Concentration of dye [mM]	Emission of fluorophor [fluorescence counts/s]
None (only cuvette)	--	3,300
None (cuvette + GAMAPC)	--	126,000
GAMAPC(bonded.) + Brilliant Blue FCF[5]	0.02	115,000
GAMAPC(bonded) + Brilliant Blue FCF[5]	0.04	94,000
GAMAPC(bonded) + Brilliant Blue FCF[5]	0.08	74,000
GAMAPC(bonded) + Brilliant Blue FCF[5]	0.16	56,000
GAMAPC(bonded) + Brilliant Blue FCF[5]	0.32	42,000
GAMAPC(bonded) + Brilliant Blue FCF[5]	0.63	29,000
GAMAPC(bonded) + Brilliant Blue FCF[5]	1.25	19,000
GAMAPC(bonded) + Brilliant Blue FCF[5]	2.50	12,000
GAMAPC(bonded) + Brilliant Blue FCF[5]	5.0	9,000
GAMAPC(bonded) + Brilliant Blue FCF[5]	10.0	8,000

Note: 5) Brilliant Blue FCF (Erioglaucine A), Fluka, Buchs, Switzerland.

A reduction in evanescence field excitation of the bonded APC that depended on the concentration of the dye in the volume was found. With a concentration of Brilliant Blue of 0.04 mM, roughly 35% of the bonded fluorescence is suppressed, i.e., the reduction in bonded fluorescence is much smaller than the reduction in fluorescence by volume excitation, where more than 95% of the fluorescence was suppressed by adding dye in the same concentration.

Example 6

This example shows that the emission of fluorophors, which are bonded to the surface, is not inhibited much by the amount of dye added, while the volume excitation is sharply reduced. The result is a better signal/noise ratio and hence lower detection limits. A cuvette prepared as in Example 1a) is placed in contact with GAMAPC in PBS+T over night at RT and then washed five times with PBS. Then, as in Example 1b), GAMAPC in PBS+T was left over night at RT on the surface. Then, it was washed five times with PBS.

The cuvettes prepared in this way went through the following different fluorescence measurements:

(1) After PBS+T was added (only bonded fluorophor)

(2) After APC was added (10 µg/ml in PBS+T) (bonded fluorophor + fluorophor in volume, but without dye)

(3) After APC 10 µg/ml and Brilliant Blue FCF (BB FCF) (0.25 mM in PBS+T) (bonded fluorophor + fluorophor in volume + dye) are added.

Table 6

Chip	Mab µg/ml	GAMAPC µg/ml	Emission [Fluorescence Counts / s]		
			(1) PBS+T	(2) PBS+T APC 10 µg/ml	(3) PBS+T APC 10 µg/ml BB FCF 0.25 mM
M1	0	10	3,000	230,000	5,000
M2	5	10	120,000	300,000	59,000
M3	5	3	72,000	280,000	29,000
M4	5	1	26,000	170,000	16,000
M5	5	0.3	5,500	230,000	7,200
M6	5	0.1	4,600	230,000	6,000

Table 7

Chip	Signal/noise Ratio[6]	
	(2) without Brilliant Blue FCF	(3) with Brilliant Blue FCF
M1[7]	--	--
M2	1.3	11.8
M3	1.2	5.8
M4	0.7	5.8
M5	1.0	3.2
M5	1.0	1.2

Notes:

6) Signal/noise ratio = ratio of surface emissions ("signal") to volume emissions ("noise")

7) Noise = Chip M1 – negative reaction (negative control).

Results:

1. Decreasing concentration series GAMAPC from M2 to M6. The negative control M1 has an emission of 3,000 counts/s.
2. Without the addition of a dye, i.e., with APC excitation in the volume not suppressed, no clear decreasing concentration series can be seen from M2 to M6. Above all, small values disappear in the background of the volume excitation.
3. With Brilliant Blue FCF in the volume, the decreasing series of concentrations, M2 to M6, can be clearly seen. The negative control M1 has 5,000 counts/s. Due to Brilliant Blue FCF, the emission of the volume by APC is reduced from 230,000 counts/s M1 to 5,000 counts/s.

The specific surface-bonded fluorescence is reduced by roughly 50%. The signal/noise ratio is much improved by the addition of Brilliant Blue FCF.

Example 7

This example measured the reaction kinetics of absorption of a fluorophor-marked protein on a reaction partner bonded to the surface of the cuvette.

In a cuvette prepared as in Example 1a), GAMAPC in PBS+T was added, and the fluorescence was measured depending on the time.

Figure 7 shows the change in emission against the time. The addition of GAMAPC was at T = 100 s. An increase in emission (fluorescence count) was observed with the reaction time, which corresponded to the absorption of the fluorophor-marked protein on the reaction partner bonded to the surface.

For comparison, the change in emission with time was measured on a sample, in which the surface of the cuvette was not coated with mouse-1gG as in Example 1a (see Fig. 8). The emission did not increase with time, but remained stable.

AMENDED CLAIMS PER INTERNATIONAL PRELIMINARY EXAMINATION REPORT DATED 26 NOVEMBER 2001

Claims

1. A method of assaying substances that includes the following steps:

- providing a surface that has at least one reaction partner R1 bonded to a surface

- placing in contact with the surface a solution that contains at least the substance being assayed, at least one compound containing a fluorophor and at least one dye that absorbs in the absorption and/or emission range of the fluorophor,
  wherein a complex forms on reaction partner R1 on the surface and wherein this complex contains, besides reaction partner R1 at least the substance being assayed and the compound containing at least one fluorophor, and

- exciting the fluorophor bonded to the surface by the evanescence field of a light source and measuring the fluorescence produced.

2. The method according to Claim 1, wherein the substance being assayed, as reaction partner R1, bonds to reaction partner R2 on the surface.

3. The method according to Claim 2, wherein the reaction partner R1 bonded to the surface is an antigen or an antibody.

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4. The method according to Claim 1, wherein a reaction partner R2 contains the substance being assayed and bonds to reaction partner R1 on the surface.

5. The method according to Claim 1, wherein another compound, which contains a bonding site for the substance being assayed and a reaction partner R2, bonds to reaction partner R1 on the surface.

6. The method according to Claim 5, wherein reaction partner R1 includes avidin or streptavidin and reaction partner R2 includes biotin and a binding site for the substance being assayed.

7. The method according to any one of the preceding claims, wherein the substance being assayed includes a biologically active substance, which is selected from the group of hormones, proteins, viruses, bacteria, pharmaceuticals and toxins.

8. The method according to any one of the preceding claims, wherein the substance being assayed includes a protein, preferably an antigen or an antibody.

9. The method according to any one of the preceding claims, wherein the compound containing fluorophor has a fluorescing compound and a binding site for the substance being assayed.

10. The method according to any one of the preceding claims, wherein fluorescing proteins and/or low-molecular fluorescing chemical compounds are used as the fluorophor.

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11. The method according to Claim 10, wherein phycobili proteins, such as allophycocyanine (APC), Cryptofluor Crimson or Cryptofluor Red are used as fluorescing proteins.

12. The method according to Claim 11, wherein Cy5 or BODIFY are used as low-molecular fluorescing compounds.

13. The method according to any one of the preceding claims, wherein at least one fluorophor that absorbs in a wavelength range from 600 to 700 nm is used.

14. The method according to any one of the preceding claims, wherein at least one phosphorescing compound is used as the fluorophor.

15. The method according to any one of the preceding claims, wherein a mixture of dyes that absorb in the absorption and/or emission range of the fluorophor is used.

16. The method according to any one of the preceding claims, wherein at least one dye that absorbs in a wavelength range form 600 to 700 nm is used.

17. The method according to Claim 16, wherein Brilliant Blue FCF in a concentration of at least 0.001 mM is used as the at least one dye.

18. Cuvette or microtiter plate for use in the method according to Claims 1 to 17 that have at least one reaction partner for the substance being assayed bonded to a surface, whereby the cuvette contains a plastic.

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19. The cuvette or microtiter plate according to Claim 18, whereby the at least one reaction partner R1 comes in lyophilized form.

20. The cuvette or microtiter plate according to Claim 18 or 19, whereby the cuvette includes polystyrene, polypropylene, polyethylene, polyacrylnitrile, polymethylmethacrylate, polycycloolefin, polyethylene terephthalate and/or mixtures thereof.

21. The cuvette or microtiter plate according to any one of Claims 18 to 20, whereby the cuvette or microtiter plate is one-piece.

22. The cuvette according to any one of Claims 18 to 21, whereby the cuvette has a reaction volume of 1 to 400 µl.

23. A solution containing at least one compound containing fluorophor, at least one dye and, if necessary, a reaction partner R2 for use in the method according to any one of Claims 1 to 17.

24. A kit for use in the method according to any one of Claims 1 to 17, including at least one cuvette or microtiter plate according to any one of Claims 18 to 22, and/or at least one solution according to Claim 23.

25. The use of the method according to any one of Claims 1 to 17 to determine reaction kinetics of immunologic reactions.

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26. The use of the method according to any one of Claims 1 to 17 in medical or veterinary medical diagnostics, food analysis, environmental analysis or analysis of fermentation processes.

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(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

(19) Weltorganisation für geistiges Eigentum
Internationales Büro

(43) Internationales Veröffentlichungsdatum
1. März 2001 (01.03.2001)

PCT

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18. August 2000 (18.08.2000)

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99116418.7 20. August 1999 (20.08.1999) EP

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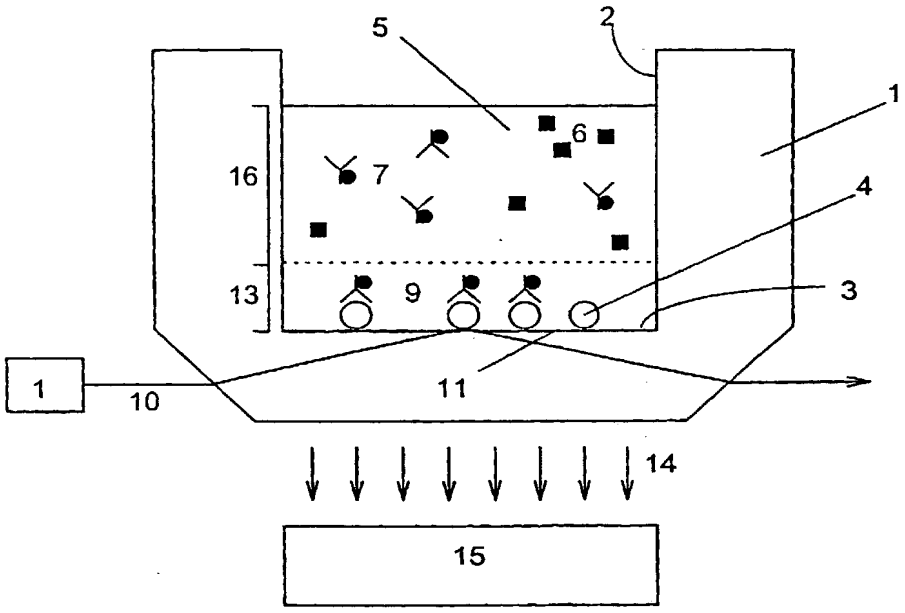
[Fortsetzung auf der nächsten Seite]

(54) Title: METHOD FOR THE DETERMINATION OF SUBSTANCES USING THE EVANESCENCE FIELD METHOD

(54) Bezeichnung: VERFAHREN ZUR BESTIMMUNG VON SUBSTANZEN MITTELS DER EVANESZENZFELDMETHODE



(57) Abstract: This invention relates to a method for the determination of substances using the evanescence field method. A cuvette, a microtiter well, a solution and a kit for application in a method according to the invention are disclosed. This method can in particular be used in the field of diagnostics and in analytical procedures.

[Fortsetzung auf der nächsten Seite]

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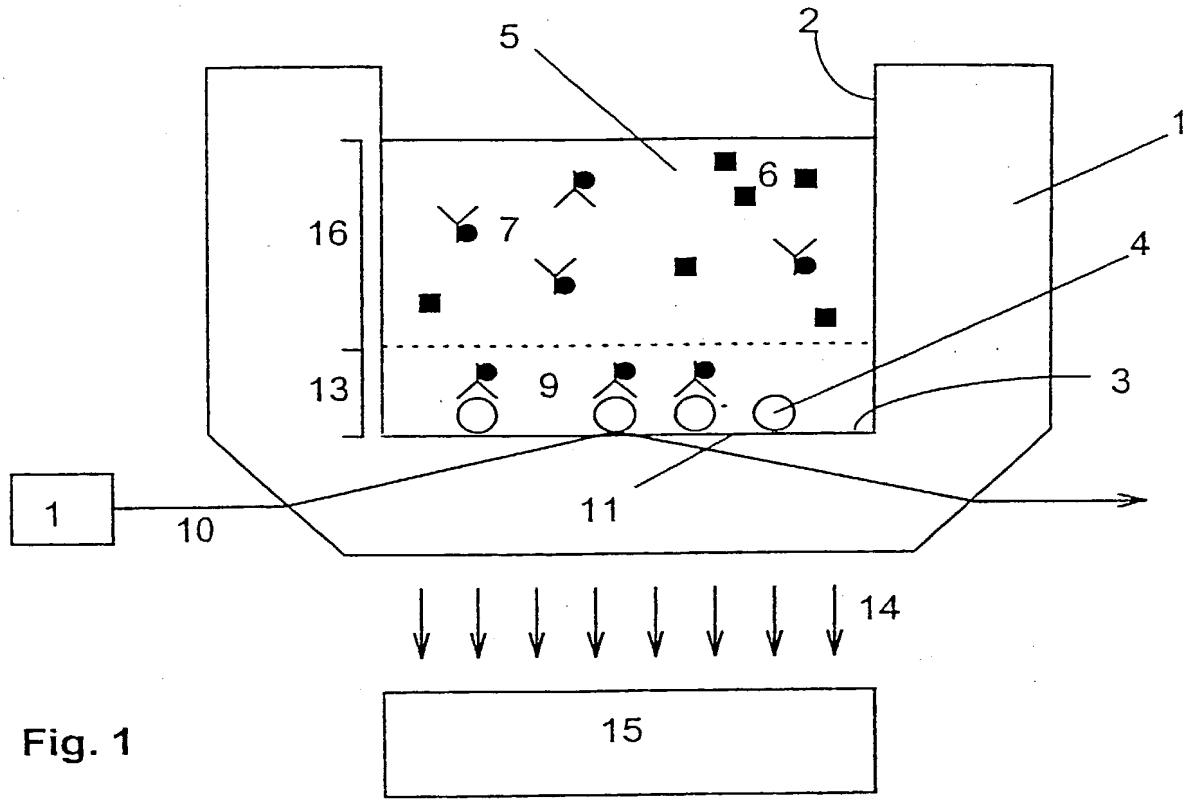

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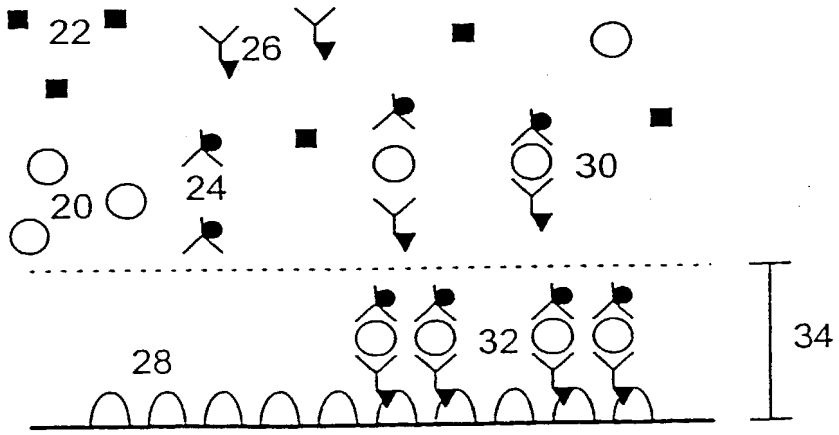

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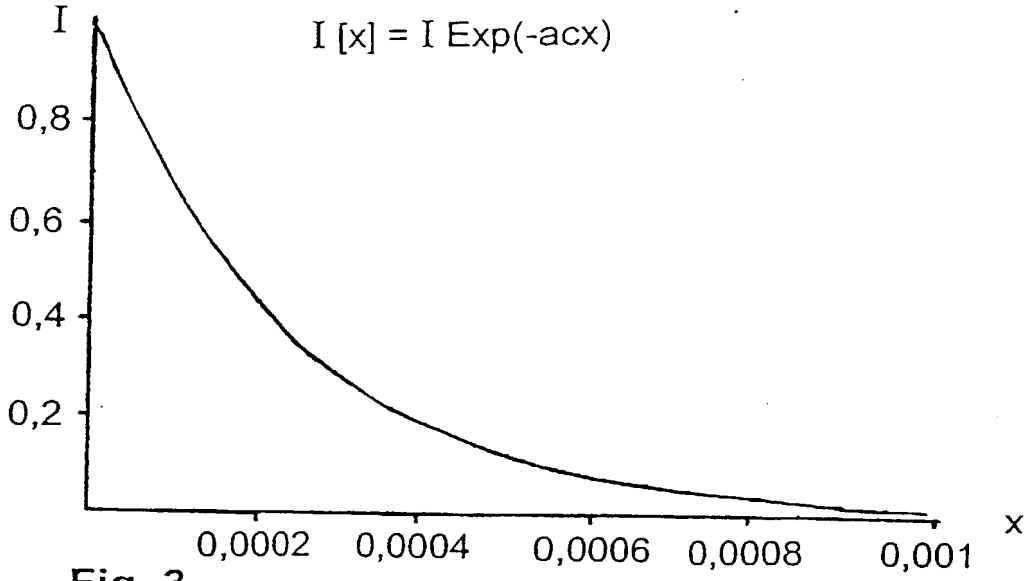
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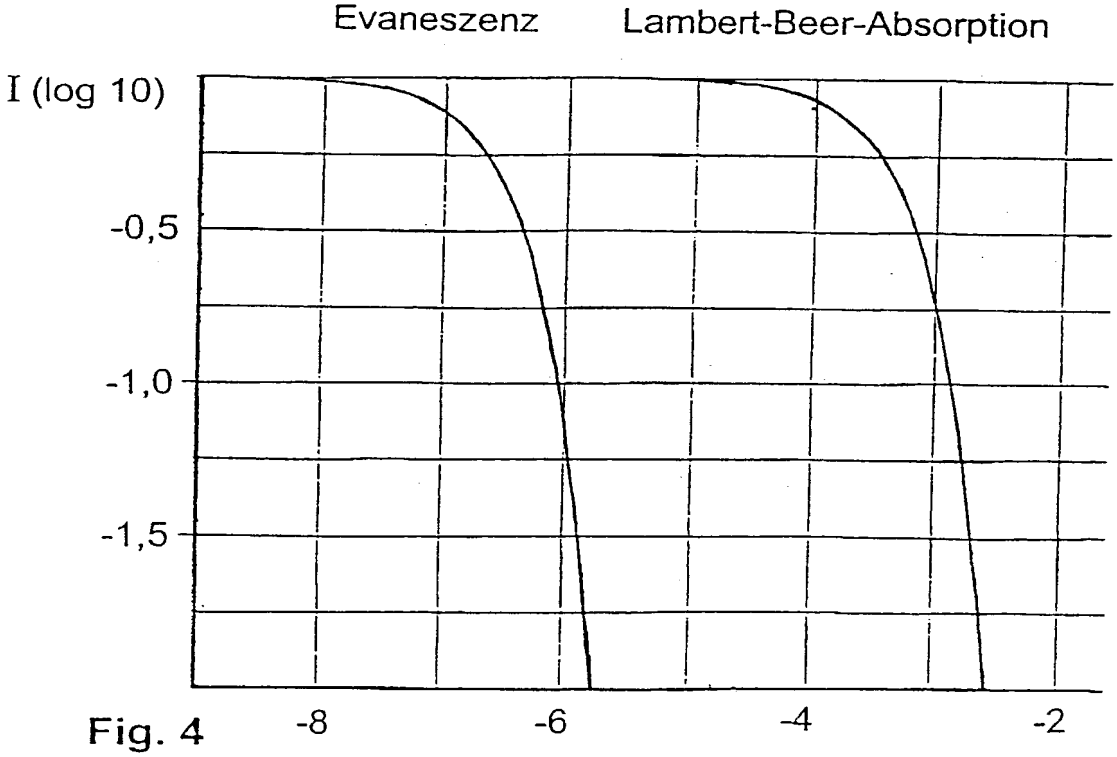
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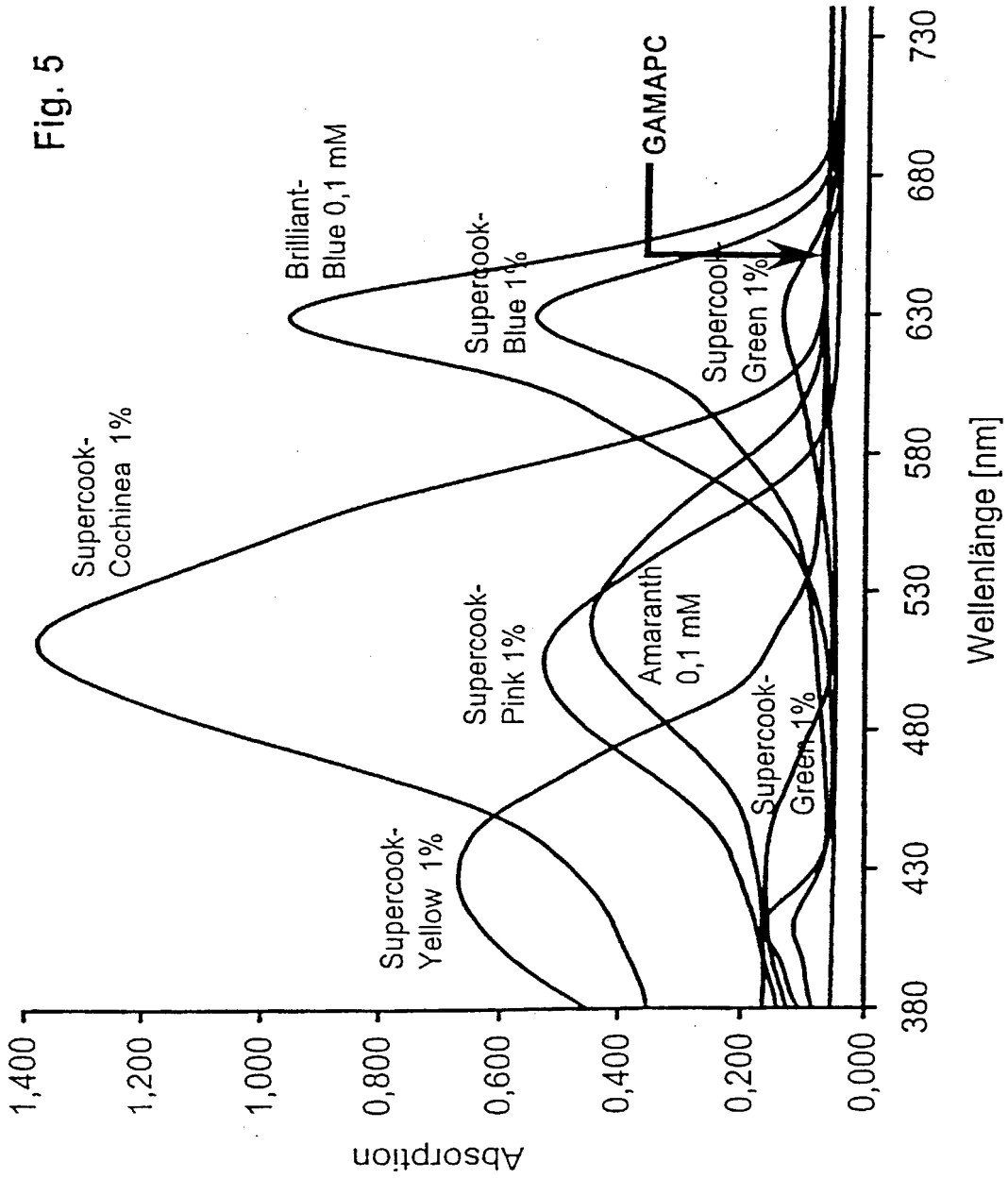
Fig. 4

Fig. 5



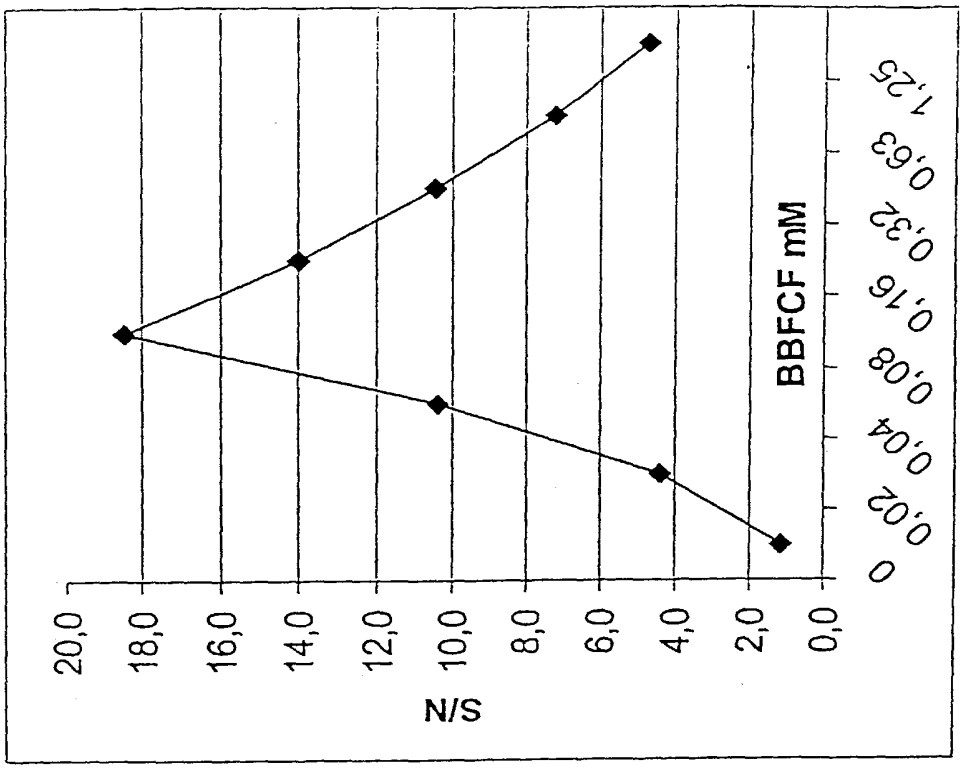

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Fig. 6b

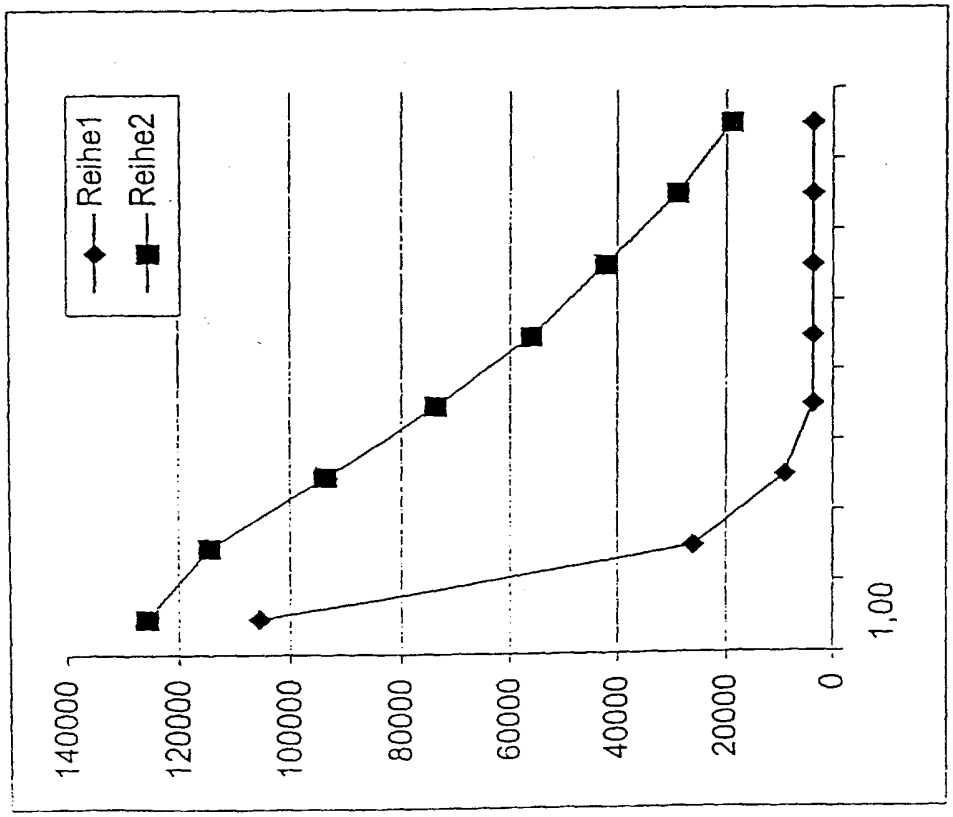
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Fig. 6a

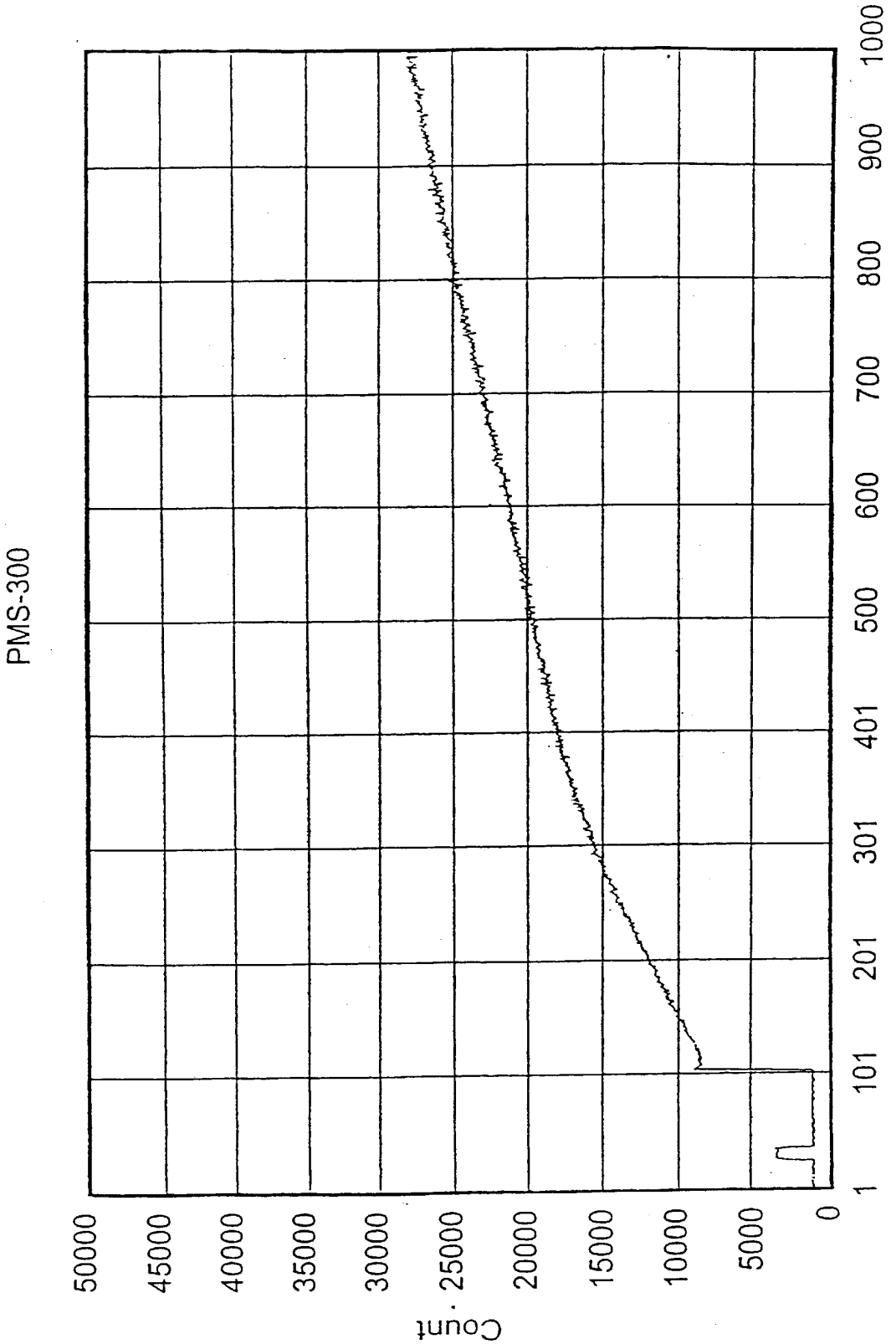
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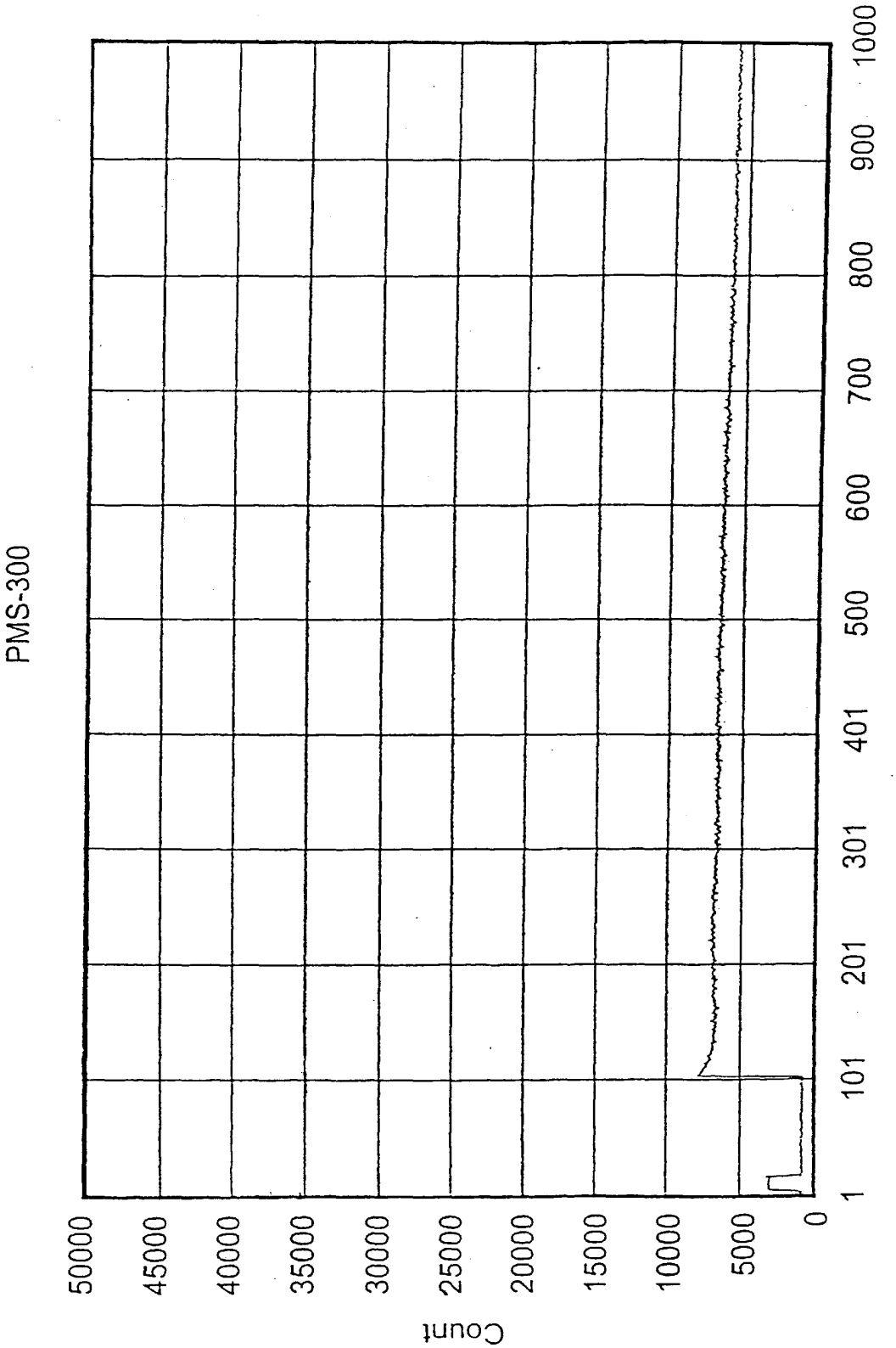
Fig. 7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Time [s]

Fig. 8

10/01

Sheet 1 of 3 D2891-PY

Attorney
Docket No.: MBP-009XX

DECLARATION AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHOD FOR THE DETERMINATION OF SUBSTANCES USING THE EVANESCENCE FIELD METHOD

The specification of which (check one):

[ ] **is attached hereto.** [X] **was filed on** 2/19/2002 **as Application No.** 10/049,975 **;**
amended on _____ **(if applicable).**

[X] was filed as PCT International. Appl. No. PCT/EP00/08116 on 18 August 2000,
and was amended under PCT Article 19 on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations §1.56(a).

I hereby claim foreign priority benefits under Title 35, USC §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)		Date Filed	Priority Claimed	
99116418.7	Europe	20 August 1999	[X]	[ ]
(Number)	(Country)	(Day/Month/Year)	Yes	No
_____	_____	_____	[ ]	[ ]
(Number)	(Country)	(Day/Month/Year)	Yes	No
_____	_____	_____	[ ]	[ ]
(Number)	(Country)	(Day/Month/Year)	Yes	No

I hereby claim the benefit under Title 35, USC §119(e) of any United States provisional application(s) listed below:

_____ _____
(Application Number) (Filing Date)

_____ _____
(Application Number) (Filing Date)

_____ _____
(Application Number) (Filing Date)

Express Mail Number

EV 009948494 US

[redacted]

Full Name of Second/Joint Inventor: Gerald Quapil		
2-00		
City of Residence ~~Owen/Teck~~	State or Country Germany DEK	Country of Citizenship Germany
Post Office Address Rebenweg 1	City 73277 Owen/Teck	State or Country Zip Code Germany
Signature: (Please sign and date in permanent ink.) X Gerald Quapil		**Date signed:** X 02. February 2002

CLG/mc/267072-1

I hereby claim the benefit under Title 35 USC §120 of any United States application(s) listed below and insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35 USC §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application No.)	(Filing Date)	(Patented/pending/abandoned)
(Application No.)	(Filing Date)	(Patented/pending/abandoned)
(Application No.)	(Filing Date)	(Patented/pending/abandoned)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) to prosecute this application and transact all business connected therewith in the Patent and Trademark Office, and to file with the USRO any International Application based thereon.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Post Office Address Au Village	City CH-1785 Cressier	State or Country Zip Code Switzerland
Signature: (Please sign and date in permanent ink.) X M. Schwall 24 Febr 2002		**Date signed:** X 24 February 2002